Quantitative Intact Proteomics Investigations of Alveolar Macrophages in Sarcoidosis

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Material and Methods

Protein fractionation

AMs were re-suspended in 1 ml homogenization buffer 4C (0.1 M potassium phosphate pH 7.4; 0.25M sucrose; and EDTA-free protease inhibitor). The samples were sonicated 4 x10 s at 35% power (Sonoplus Ultrasonic homogenizer). The sample solutions were then ultra-centrifuge at 100 000 X g for 1h (XL-70 Ultracentrifugation, Beckman^{TM} 50.4 Ti rotor) to obtain soluble and membrane fractions. The protein concentrations of the soluble fractions were determined using the method of Bradford (1).

Proteomics analysis using minimal DIGE

Aliquots of 50μg protein were labeled with 400 pmol minimal DIGE according the manufacturer's recommendations. The Cy2 label was assigned to the pooled internal standard in all 2DE gels. Cy3 and Cy5-dyes were randomly assigned to samples of healthy and sarcoidosis patients. The protein labeling occurred for 30 min in the dark, and was then quenched by 10 mM fresh lysine solution. Samples were loaded onto 24 cm IPG-strip pH (4-7 linear) for in-gel rehydration overnight. Isoelectric focusing (IEF) was then performed using Ettan IPGphor II electrophoresis unit with a total focusing time of 14 hrs and 53.7 kVh. Strips were immediately equilibrated with 10 ml equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS) containing 1% DTT (w/v) in the 1st equilibration step, and then with 2.5% w/v iodoacetamide in the 2nd step. Finally, strips were run on SDS-PAGE (12.5 %) homogeneous gels, on the Ettan^{TM} DALT*six* electrophoresis unit overnight (2W/gel).

Image acquisition

A Typhoon-9410 fluorescence scanner was used to collect the image data from large the format 2DE gels (24cm x 20cm). All of the images were collected at 100 µm resolution at 550 Volt PMT, which corresponded to an optimized dynamic range nearly saturating the most intense spot in the gel image. The Cy2-labeled images were excited with an Argon laser at 488 nm, and an band pass emission filter of 520BP40 nm was used. Cy3-labeled images were excited with Nd:YAG laser at 532 nm and the emission collected at 580BP30 nm. Cy5-labeled images were excited with Helium-Neon laser at 633 nm and the emission light collected at 670 nm; BP-30 (640-700nm).

In-gel tryptic digestion

Protein-spots of interest were excised and subjected to in-gel tryptic digestion (Sequencing Grade Modified Trypsin, Promega). Gel-plugs were destained (30 mM $K_3Fe(CN)_6$:100 mM $Na_2S_2O_3$ (1:1)), equilibrated (0.05M NH_4HCO_3) and dehydrated after washes with acetonitrile (ACN). Gel plugs were re-swollen in 0.05M NH_4HCO_3 containing 200 ng Trypsin (4ºC, 30 min) following tryptic digestion overnight (37 ºC). Peptides extraction was performed twice with 0.1% TFA, 60% ACN. Zip-tip C-18 technique was used to desalts and peptide enrichment.

Matrix-assisted laser desorption ionization time of flight (MALDI-TOF)

Sample aliquotes of 1 µl (60% ACN; 0.1% TFA)were loaded together with the matrix solution CHCA (0.7 µl) onto the MALDI-plate for the MS analysis (Micromass MALDI-R TOF mass spectrometer). The setup for data acquisition was: positive mode, mass range 500-

3000 Da m/z, laser firing rate 5 Hz, 10 shots per spectrum tuned at15% of the laser energy. Internal calibration was performed using the trypsin auto-digestion peptides (842.510; 1045.564; and 2211.105 m/z). Next, the protein identification was done at the NCBInr sequence database (vs 2009/03/01) using the searching program ProFound (http://prowl.rockefeller.edu) with the following parameters applied: human database, one missed trypsin-cleavage; complete modification: Iodoacetamide (Cys); and partial oxidation of Methionine, molecular weight (10-150 kDa) and isoelectric points (4-7). Probability value <0.001, and Z-score>0.7 were the thresholds values used for protein identification.

MALDI TOF-TOF analysis

Each sample was mixed with 7mg/ml a-cyano-4-hydroxycinnamic acid (CHCA, Bio-Rad, Hercules, CA) in 70% acetonitrile before spotted to the MALDI target plate. External calibration spots with 0.5 µl of BSA tryptic digests were spotted on the plate. A 4800 MALDI TOF/TOF (Applied Biosystems, Framingham, MA, USA) instrument was used to analyse the MS and MS/MS mode. A maximum of 10 precursors with S/N over 100 was set to be picked up from each spot and 1000 shots in the range 700 – 4000 *m/z* were acquired for each MS spectrum. MS/MS was performed averaging 3000 shots. All MS/MS data were submitted to Mascot (Matrix Science, London, UK) for database searching. The searches were conducted against NCBInr human protein sequence database (version 2009/03/01). The search parameters were set to variable modifications: Oxidation (M), Deamidated (NQ), and Carbamidomethyl (C), Fragment mass tolerance: ± 0.6 Da and maximum 1 missed cleavage site. Proteins were considered to be identified if at least two peptides matched, Rank=1, Ion score >39 (significance threshold set to 95% ($p \leq 0.05$)).

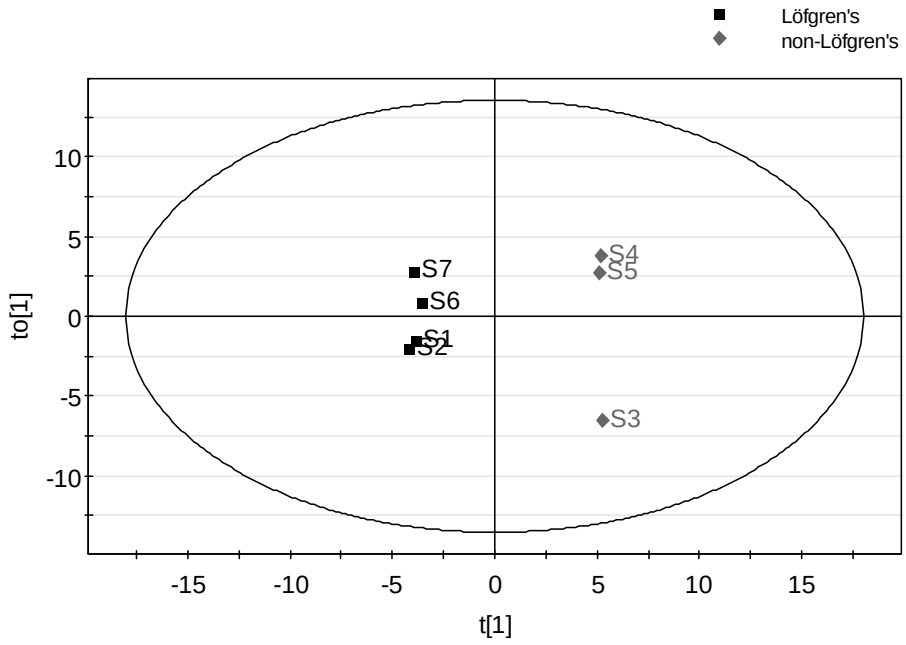

Figure S1. OPLS analysis based on sarcoidosis phenotype. The scores plot indicates a clear separation between Löfgren's (S1, S2, S6 and S7) and non-Löfgren's patients (S3, S4, S5) with a high predictive power (Q^2=0.72).

References

1. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. AnalBiochem. 1976;72:248-54.